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A New and Noteworthy Hybrid of *Viola* (Violaceae) from Iwate, Northern Japan:

Viola rostrata var. *japonica* × *V. violacea* var. *makinoi*

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A plant morphologically intermediate between *Viola rostrata* Pursh var. *japonica* (W. Becker et H. Boissieu) Ohwi and *V. violacea* Makino var. *makinoi* (H. Boissieu) Hiyama ex F. Maek. was found in Iwate Prefecture, Tohoku District, northern Japan. Molecular analysis based on sequences of internal transcribed spacer regions of nuclear ribosomal DNA and *trnL-trnF* intergenic region of chloroplast DNA indicated that the intermediate plant was a hybrid between those two species and that the maternal parent was *V. rostrata* var. *japonica*. This is the first report of a naturally occurring intersubsectional hybrid of subsect. *Rostratae* Kupffer and subsect. *Adnatae* W. Becker.

Key words: hybrid, Japan, subsection *Adnatae*, subsection *Rostratae, Viola*

Viola L. (Violaceae) is a well-known genus with many interspecific hybrids. Species of violets generally have weak barriers to interspecific gene flow (Conesa *et al*. 2008) and many hybrids have been reported (e.g. Becker 1910, 1917, Brainerd 1924, Hama 1975, 2002, Marcussen & Karlsson 2010). The verification of interspecific hybrids based on molecular evidence has increased (e.g. Neuffer *et al*. 1999, Conesa *et al*. 2008, Marcussen *et al*. 2011, 2012).

One of us (S. Takeda) found a morphological intermediate between *Viola rostrata* Pursh var. *japonica* (W. Becker et H. Boissieu) Ohwi and *V. violacea* Makino var. *makinoi* (H. Boissieu) Hiyama ex F. Maek. in a location where the two species coexist (Fig. 1). The main features of the intermediate plant are summarized in Table 1.

Viola rostrata has a particularly long spur (hence the common name, long-spurred violet). It occurs in eastern North America and in the Japanese archipelago adjacent to the Sea of Japan (Harvey 1966, Hashimoto 1967). In addition to the main area of distribution in Japan, isolated occurrences have been reported from the Pacific side of the Tohoku, Kanto, and Shikoku districts (Hama 1975, 2002). The Japanese populations are classified as the endemic variety, *V. rostrata* var. *japonica*, which grows primarily in the understory or on the edge of deciduous forests (Akiyama *et al*. 1999). *Viola rostrata* is a member of sect. *Viola*, subsect. *Rostratae* Kupffer (syn. sect. *Trigonocarpea* Godr.; basic chromosome number, x = 10). Species in subsect. *Rostratae* are characteristically perennial and usually caulescent herbs with a cylindrical style (Marcussen & Karlsson 2010). Approximately 50 species are included in this subsection (van den Hof *et al*. 2008), 11 of which are native to Japan. In con-

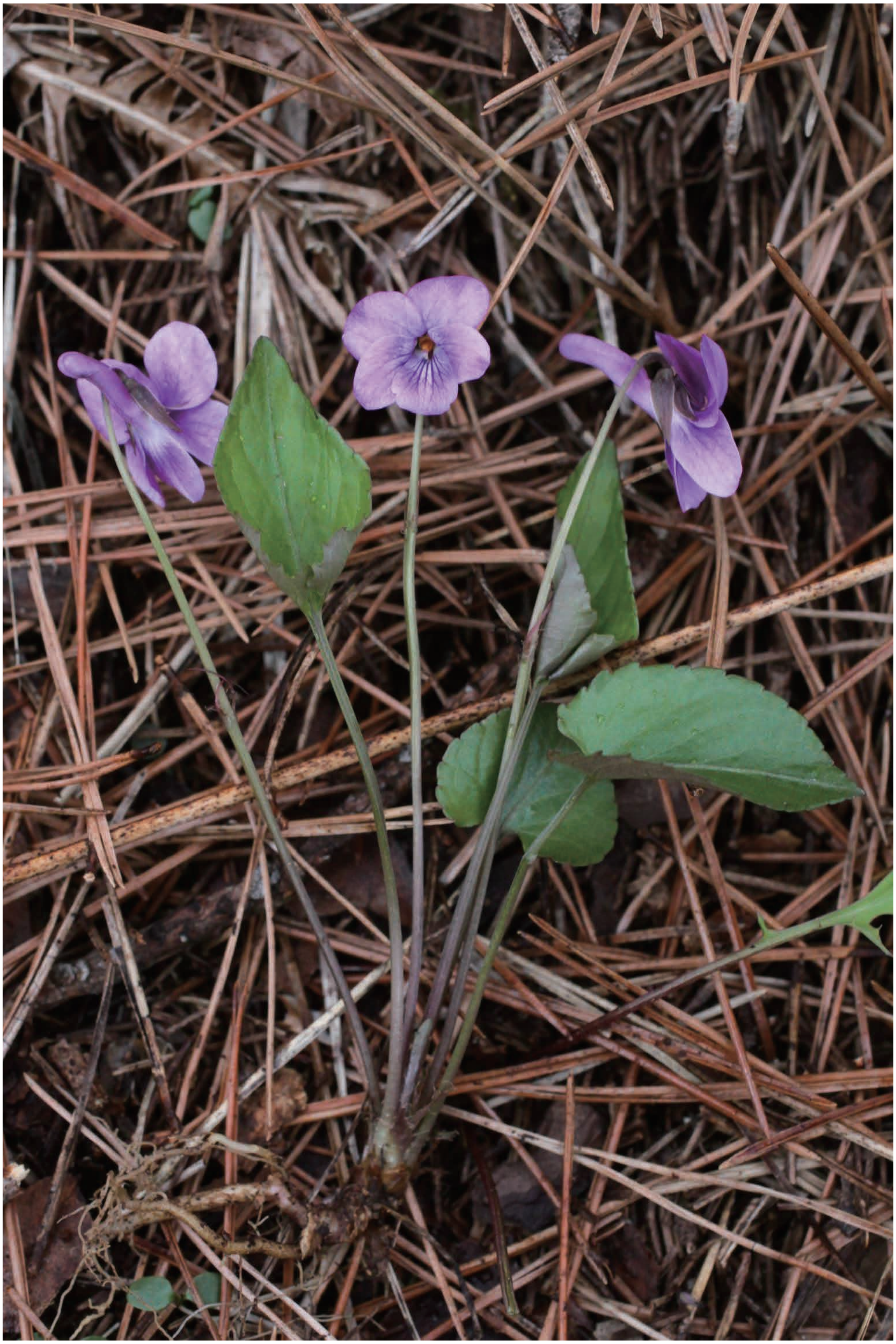

FIG. 1. *Viola rostrata* var. *japonica* × *V. violacea* var. *makinoi*.

TABLE 1. Morphological features of *Viola rostrata* var. *japonica, V. violacea* var. *makinoi* and putative hybrid individual.

	V. rostrata var. *japonica*	intermediate plant	*V. violacea* var. *makinoi*
caulis	caulescent	acaulescent without stolons	acaulescent without stolons
rosette leaf*	present	absent	absent
petal color	pale purple to reddish purple	reddish purple	reddish purple
style	cylindrical	near cylindrical; upper part slightly enlarged	like mentis head
leaf morphology	cordate	cordate	narrowly lanceolate
margin of stipules	pectinate	pectinate	entire or a with few serrattions

Features of *Viola rostrata* var. *japonica* and *V. violacea* var. *makinoi* are from Hama (2002) and Igari (2004)
*: overwintering leaves

trast, *Viola violacea* is distributed across Japan, South Korea, and China (Chen *et al.* 2007) and often grows in the understory and on the margins of deciduous or pine forests. Two varieties of *V. violacea* are distributed widely across the Japanese archipelago: *Viola violacea* var. *violacea*, with narrowly ovate to widely lanceolate leaf blades in western Japan; and *V. violacea* var. *makinoi*, with lanceolate to narrowly lanceolate leaf blades in eastern Japan (Hama 1975, 2002, Igari 2004). *Viola violacea* is a member of sect. *Plagiostigma* Godr., subsect. *Adnatae* W. Becker (syn. sect. *Violidium* K. Koch; basic chromosome number, x = 12). Species in subsect. *Adnatae* are characteristically perennial, acaulescent herbs lacking stolons, and with the stipules adnate to the petiole (Marcussen & Karlsson 2010). The shape of the style, referred to as like a head of mantis, is also characteristic of subsect. *Adnatae* (Hashimoto 1967, Igari 2004). Species in subsect. *Adnatae* are distributed mainly in eastern Asia (Marcussen and Karlsson 2010). Approximately 50 species are included in this subsection (as the "*Adnatae* group") by Nakamura *et al.* (2015); ca. 20 species are native to Japan. Several infrageneric classifications have been proposed in *Viola* (cf. Wang 1991, Ballard *et al.* 1999), but we follow that of Marcussen & Karlsson (2010) and Marcussen *et al.* (2015).

Here we report a novel and noteworthy hybrid of *Viola rostrata* var. *japonica* and *V. violacea* var. *makinoi* based on morphological and molecular comparisons. This first report of a natural hybrid between subsect. *Rostratae* and subsect. *Adnatae* will enhance our understanding of violet evolution.

Materials and Methods

Materials

A plant morphologically intermediate between two putative parental species was collected in Hanamaki City, Iwate Pref., Tohoku district, Japan (39°26'10.4" N, 141°03'32.1" E, alt. ca. 170 m) on May 2, 2012. The collection site is a southwest-facing slope located at the edge of a Japanese red pine (*Pinus densiflora* Siebold et Zucc.) forest. *Viola rostrata* var. *japonica* and *V. violacea* var. *makinoi* coexist in the ca. 20 × 10 m area. No other species of *Viola* occur at this site. To increase the accuracy of genetic comparison, we added samples from other localities; *V. rostrata* var. *japonica* from Akita City, Akita Pref., Japan (39°44'25.97" N, 140°19'32.73" E, alt. ca. 190 m) and Nishikawa Town, Yamagata Pref., Japan (38°27'42.24" N, 139°59'57.81" E, alt. ca. 550 m) and sequences of *V. violacea* var. *makinoi* from the EMBL/DDBJ/GenBank International DNA Database. The number of samples used for morphological measurements and molecular analyses are listed in Table 2. We resurveyed the same site on April 26, 2013 to ascertain the existence of other intermediate plants, but no additional individuals were found.

Voucher specimens for this study were depos-

TABLE 2. Sampling localities and number of individuals used comparison.

		Number of individuals		Accession number	
species	Locality	Morphological measurement	Molecular comparison	ITS	*trnL-trnF*
Viola rostrata var. *japonica*	Hanamaki City, Iwate Pref., Japan	20	2	LC079041, LC079042	LC079049, LC079050
	Nishikawa Town, Yamagata Pref., Japan	—	1	LC079043	LC079051
	Akita City, Akita Pref., Japan	—	1	LC079044	LC079052
V. violacea var. *makinoi*	Hanamaki City, Iwate Pref., Japan	20	2	LC079045, LC079046	LC079053, LC079054
	Yamagata City, Yamagata Pref., Japan*	—	1	AB759254	AB759279
	Sendai City, Miyagi Pref., Japan*	—	1	AB759252	AB759277
intermediate plant**	Hanamaki City, Iwate Pref., Japan	1	1	LC079047, LC079048	LC079055

*: Yoshida *et al.* (2013)
**: An identical individual was used for measurements and molecular analysis

ited in the herbarium of Yamagata Prefectural Museum, Yamagata (YAMA) under the following reference numbers: *Viola rostrata* var. *japonica* × *V. violacea* var. *makinoi* in Hanamaki, Iwate Pref., *S. Takeda 778 (YAMA 74577)*; *V. rostrata* var. *japonica* in Hanamaki, Iwate Pref., *S. Takeda 777 (YAMA 74576)*, in Akita, Akita Pref., *M. Yoshida 12098 (YAMA 74592)*, in Nishikawa, Yamagata Pref., *M. Yoshida & J. Yokoyama 12100 (YAMA 74593)*; *V. violacea* var. *makinoi* in Hanamaki, Iwate Pref., *S. Takeda 776 (YAMA 74575).*

Morphological comparison of flowers and leaves

The largest flower and leaf from each of the individuals at the time of collection were selected for measurement. Three flowers and four leaves from a single individual of the intermediate plant were measured. Three floral characters were measured: corolla width (horizontal width of the corolla), spur length (length from the joint of the pedicel to the tip of the spur), and lip length (length from the joint of the pedicel to the tip of the lip). Two leaf characters were measured: leaf blade length and width. We also calculated the ratio between the spur and lip length (spur/lip) and the ratio between the leaf blade length and width (length/width). These measurements and ratios were tested using the Kruskal-Wallis nonparametric ANOVA followed by the post-hoc Steel-Dwass method using R version 3.0.2 software (R Core Team 2013).

Molecular analysis

Total DNA was extracted from leaves that were dried in silica gel using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. We amplified the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) using the universal primers ITS5 and ITS4 (White *et al.* 1990) and amplified the *trnL-trnF* intergenic spacer region of chloroplast DNA (cpDNA) using "e" and "f" primers (Taberlet *et al.* 1991) using the PCR program of Yoshida *et al.* (2013). The amplified products were purified using a QIAEXII Gel Extraction Kit (Qiagen, Hilden, Germany). Cycle sequencing was performed using a BigDye Terminator Cycle Sequencing Kit v1.1 (Applied Biosystems, Foster City, CA, USA) and the products were purified using an illustra Autoseq G-50 Dye Terminator Remover Kit (GE Healthcare, Milwaukee, WI, USA). The sequences were determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For the intermediate plant, the amplified products of the ITS region were purified as described above and cloned using the TA Cloning Kit, with PCR2.1 Vector and One Shot TOP10 Chemically

TABLE 3. Haplotypes of nrDNA ITS and *trnL-trnF* intergenic region of cpDNA.

species	sampling locality	ITS type	*trnL-trnF* type
V. rostrata var. *japonica*	Hanamaki	nrR-1, nrR-2	cpR-1
	Nishikawa	nrR-2 (nrR-4)	cpR-1
	Akita	nrR-3	cpR-2
V. violacea var. *makinoi*	Hanamaki	nrM	cpM (cpM-1)
	Sendai	nrM	cpM (cpM-1)
	Yamagata	nrM	cpM (cpM-2)
hybrid individual	Hanamaki	nrR-1 / nrM	cpR-1

Haplotypes when the gaps are treated as missing data are shown (ITS: 629 bp, *trnL-trnF*: 375 bp). The ID in the parentheses are when the gaps are treated as fifth state (ITS: 657 bp, *trnL-trnF*: 389 bp).

Competent *E. coli* (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. This additional step was done because of many overlapping peak patterns and unreadable results from direct sequencing.

DNA sequences were aligned using MUSCLE (Edgar 2004) in MEGA v.5.05 software (Tamura *et al*. 2011). TCS v1.21 software (Clement *et al*. 2000) was used to determine the haplotype identity of each sequence.

Results and Discussion

Comparison of the internal transcribed spacer (ITS) region sequences revealed that the intermediate plant had two haplotypes which matched those of *Viola rostrata* var. *japonica* and *V. violacea* var. *makinoi* collected in Hanamaki (Table 3). Therefore, we considered the intermediate plant to be a hybrid of the two taxa. The *trnL-trnF* sequence of the hybrid individual indicated that *V. rostrata* var. *japonica* was the maternal parent because the chloroplast DNA (cpDNA) of violets is the product of maternal inheritance (Harris & Ingram 1991).

The stem, rosette leaves, and petal color of the hybrid individual resembled those of *Viola violacea* var. *makinoi*, whereas the leaves and stipules resembled those of *V. rostrata* var. *japonica* (Table 1). The style morphology of the hybrid was intermediate between the two taxa (Fig. 2). Hama (2002) noted that hybrids of violets with aerial stems (caulescent or acaulescent with stolons) and without aerial stems (acaulescent without stolons) have no aerial stems. The hybrid individual we discovered was acaulescent and without stolons and its morphology matched observations of other hybrids. When hybridization occurs between individuals with and without aerial stems, the latter may be the dominant character, which was the case in this instance.

Morphological measurements are shown in Table 4. The corolla of *Viola rostrata* var. *japonica* was wider than in the hybrid individual and in *V. violacea* var. *makinoi.* The spur length and spur/lip ratio, from long to short, were: *V. rostrata* var. *japonica*, hybrid individual, *V. violacea* var. *makinoi*. The leaves of *V. violacea* var. *makinoi* were narrower than those of *V. rostrata* var. *japonica* and the hybrid. Although no statistically significant difference was detected between *V. rostrata* var. *japonica* and the hybrid, the latter appeared to have narrower leaves. Measurements of the hybrid as a whole were similar to those of *V. rostrata* var. *japonica*.

Most hybrids of *Viola* are sterile or have lower fertility than pure species, irrespective of whether crosses are between closely or distantly related species (Schmidt 1961, Harvey 1966, Hashimoto 1967, Hama 1975, 2002, Hodálová *et al*. 2008). To avoid destroying the specimen, we did not observe a sufficient number of pollen grains of the hybrid, but those we did inspect were deformed and smaller than those of the parental species, suggesting possible sterility of the hybrid (data

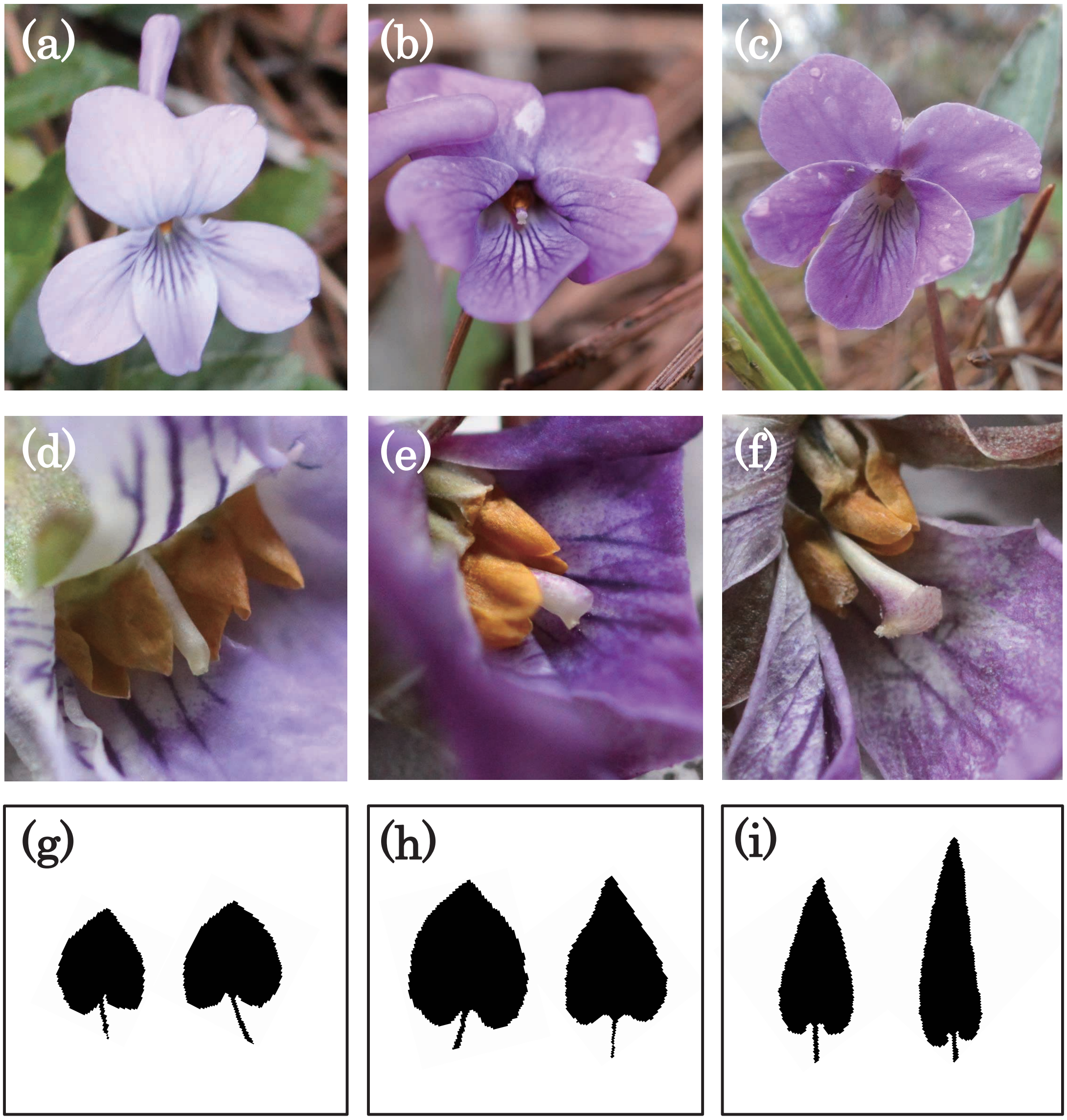

FIG. 2. Flower (a—c), style (d—f) and silhouette of leaf blade (g—i). *Viola rostrata* var. *japonica* (a, d, g), *V. rostrata* var. *japonica* × *violacea* var. *makinoi* (b, e, h) and *V. violacea* var. *makinoi* (c, f, i)

not shown). The integrity of the species of violets may be retained through postzygotic isolation, specifically through hybrid sterility.

The relationship of the subsections to which the parental species of hybrids belong is interesting. When varieties and forms are considered, more than 120 intrasubsectional hybrids in subsect. *Adnatae* and 20 in subsect. *Rostratae* have been reported in Japan (Hama 2002). This profusion of hybrids could be explained by the abundance of species in those two subsections and to studious and careful observations by botanists and violet fanciers. Moreover, several natural and artificial intersectional / subsectional hybrids with a parent from subsect. *Adnatae* have been reported. They include hybrids within sect. *Plagiostigma*; subsect. *Vaginatae* W. Becker, subsect. *Diffusae* W. Becker, (Hama 1975, 2002), subsect. *Bilobatae* W. Becker (Hama 2002), and subsect. *Stolonosae* Kupffer (Becker 1917, Ger-

TABLE 4. Results of measuements and ratios.

	V. rostrata var. *japonica* ($N = 20$)	Hybrid individual ($N = 1$)*	*V. violacea* var. *makinoi* ($N = 20$)
Measurements (mm)			
corolla width	19.35 ± 2.62 (15–23, 20^{a})	15.00 ± 2.65 (13–18, 14^{ab})	16.80 ± 1.20 (15–18, 17^{b})
spur length	14.05 ± 0.89 (13–16, 14^{a})	9.67 ± 1.53 (8–11, 10b)	7.50 ± 1.54 (5–11, 7^{b})
lip length	13.20 ± 1.40 (11–16, 13)	12.67 ± 1.53 (11–14, 13)	12.35 ± 1.35 (10–15, 12)
leaf blade length	21.90 ± 4.13 (15–32, 21^{a})	22.25 ± 3.77 (17–25, 23.5^{a})	34.40 ± 5.81 (23–44, 35^{b})
leaf blade width	19.15 ± 3.76 (13–27, 19^{a})	17.75 ± 2.06 (16–20, 17.5^{a})	11.85 ± 2.30 (7–15, 12^{b})
Ratios			
spur / lip length	1.07 ± 0.06 (0.94–1.18, 1.08^{a})	0.76 ± 0.03 (0.73–0.79, 0.77^{b})	0.60 ± 0.08 (0.45–0.73, 0.58^{c})
leaf blade length/width	1.15 ± 0.12 (0.91–1.33, 1.16^{a})	1.25 ± 0.14 (1.06–1.38, 1.29^{a})	2.93 ± 0.31 (2.53–3.71, 2.92^{b})

Numerical values indicate mean ± SE with ranges. Each value of the minimum-muximum and median are shown in parenthesis. Median superscript indicate statistical significant differences determined post hoc Steel-Dwass test ($p < 0.05$).
*: Three flowers and four leaves were collected from an intermediate plant.

shoy 1934). A few artificial hybrids have been bred between species in sect. *Viola* subsect. *Viola* (*V. mandshurica* W. Becker × *V. odorata* L., *V. violacea* var. *violacea* × *V. odorata*). In contrast, intersectional / subsectional hybrids of subsect. *Rostratae* with subsect. *Viola* (Becker 1910, Hama 1975, 2002, Marcussen & Borgen 2000), subsect. *Stolonosae* and sect. *Nosphinium* W. Becker subsect. *Boreali-Americanae* (W.Becker) Gil-ad (Gershoy 1934) have been recorded. Although artificial hybridization experiments for North American violets were conducted by Gershoy (1934), seeds of putative hybrid origin between subsect. *Rostratae* (as sect. *Nomimium* Ging. subsect. *Rostellatae* Boissieu; *V. adunca* Sm.) and subsect. *Adnatae* (as sect. *Nomimium* subsect. *Plagiostigma* group *Adnatae*; *V. pinnata* L.) were obtained, but did not germinate. Thus, although several instances have been reported as described above, naturally occurring hybrids between species of subsect. *Rostratae* and subsect. *Adnatae* have not yet been reported. Considering that coexistence of the taxa of these two subsections is common in Japan, the absence of hybrids between species in those subsections may indicate that they have simply been overlooked due to low hybridization frequency. The details of the physiological and genetic mechanisms of hybridization in violets are unclear. The incidence described here is rare, but is valuable for elucidating violet evolution.

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References

Akiyama, S., H. Ohba & S. Tabuchi. 1999. Violaceae. *In*: Iwatsuki K., D. E. Boufford and Ohba H. (eds.) Flora of Japan, Vol. IIc. pp. 161–190. Kodansha, Tokyo.

Ballard H. E., K. J. Sytsma & R. R. Kowal. 1999. Shrinking the violets: Phylogenetic relationships of infrageneric groups in *Viola* (Violaceae) based on internal transcribed spacer DNA sequences. Syst. Bot. 23: 439–458.

Becker, W. 1910. Violae Europaeae. Systematische Bearbeitung der Violen Europas und seiner benachbarten Gebiete. C. Heinrich, Dresden.

Becker, W. 1917. Violae Asiaticae et Australenses. I. Beih. Bot. Centralbl. Abt. 2. 34: 208–266.

Brainerd, E. 1924. Some natural violet hybrids of North America. Vermont Agricultural Experiment Station Bulletin 239.

Chen, Y. S., Q. E. Yang, H. Ohba & VI. V. Nikitin. 2007. Violaceae. *In*: Wu, Z. Y., P. H. Raven & D. Y. Hong (eds.), Flora of China. Vol. 13 (Clusiaceae through Araliaceae). pp. 72–138. Science Press, Beijing and Missouri Botanical Garden Press, St. Louis.

Clement, M., D. Posada & K. A. Crandall. 2000. TCS: a computer program to estimate gene genealogies. Molec. Ecol. 9: 1657–1659.

Conesa, M. À., M. Mus & J. A. Rosselló. 2008. Hybridization between insular endemic and widespread species of *Viola* in non-disturbed environments assessed by nuclear ribosomal and cpDNA sequences. Plant. Syst. Evol. 273: 169–177.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32: 1792–97.

Gershoy, A. 1934. Studies in North American violets III. Chromosome numbers and species characters. Vermont Agric. Exp. Sta. Bull. 367: 3–91.

Hama, E. 1975. The Wild Violets of Japan in Color. Seibundo-Shinko-sha. Tokyo (in Japanese).

Hama, E. 2002. Enlarged and Revised Edition of The Wild Violets of Japan in Color. Seibundo-Shinko-sha. Tokyo (in Japanese).

Harris, S. A. & R. Ingram. 1991. Chloroplast DNA and biosystematics: the effects of intraspecific diversity and plastid transmission. Taxon 40: 393–412.

Harvey, M. J. 1966. Cytotaxonomic relationships between the European and North American rostrate violets. New Phytologist 65: 469–476.

Hashimoto, T. 1967. The violets of Japan. Seibundo-Shinko-sha. Tokyo (in Japanese).

Hodálová, I., P. Mered'a Jr., P. Mártonfi, L. Mártonfiová & J. Danihelka. 2008. Morphological characters useful for the delimitation of taxa within *Viola* subsect. *Viola* (Violaceae): A morphometric study from the West Carpathians. Folia Geobot. 43: 83–117.

Igari, M. 2004. Enlarged and Revised Edition of Wild Violets of Japan. Yama-to-Keikoku-sha. Tokyo (in Japanese).

Marcussen, T. & L. Borgen. 2000. Allozymic variation and relationships within *Viola* subsection *Viola* (Violaceae). Plant Syst. Evol. 223: 29–57.

Marcussen, T. & T. Karlsson. 2010. Violaceae. *In*: Jonsell, B. and T. Karlsson (eds.), Flora Nordica 6. pp. 12–52. Swedish Mus. Nat. Hist. Stockholm.

Marcussen, T., K. Blaxland, M. D. Windham, K. E. Haskins & F. Armstrong. 2011. Establishing the phylogenetic origin, history, and age of the narrow endemic *Viola guadalupensis* (Violaceae). Am. J. Bot. 98: 1978–1988.

Marcussen, T., K. S. Jakobsen, J. Danihelka, H. E. Ballard, K. Blaxland, A. Brysting & B. Oxelman. 2012. Inferring Species Networks from Gene Trees in High-Polyploid North American and Hawaiian Violets (*Viola*, Violaceae). Syst. Biol. 61: 107–126.

Marcussen, T., L. Heier, A. K. Brysting, B. Oxelman & K. S. Jakobsen. 2015. From gene trees to a dated allopolyploid network: insights from the angiosperm genus *Viola* (Violaceae). Syst. Biol. 64: 84–101.

Nakamura, K., T. Denda, G. Kokubugata, C. J. Huang, C. I. Peng & M. Yokota. 2015. Phylogeny and biogeography of the *Viola iwagawae-tashiroi* species complex (Violaceae, section *Plagiostigma*) endemic to the Ryukyu Archipelago, Japan. Plant Syst. Evol. 301: 337–351.

Neuffer, B., H. Auge, H. Mesch, U. Amarell, & R. Brandl. 1999. Spread of violets in polluted pine forests: morphological and molecular evidence for the ecological importance of interspecific hybridization. Molec. Ecol. 8: 365–377.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. < http://www.R-project.org/.>

Schmidt, A. 1961. Zytotaxonomische Untersuchungen an europäischen *Viola*-Arten der Sektion *Nomimium*. Öester. Bot. Zeitschr. 108: 20–88.

Taberlet, P., L. Gielly, G. Pautou & J. Bouvet. 1991. Universalprimers for amplification of three non-coding region of chloroplast DNA. Plant Molec. Biol. 17: 1105–1109.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei & S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molec. Biol. Evol. 28: 2731–2739.

Wang, Q. R.. 1991. *Viola*. *In*: Wang, Q. R. (ed.), Flora Reipublicae Popularis Sinicae 51. Science Press, Beijing (in Chinese).

White, T. J., T. Bruns, S. Lee & J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis, M., D. Gelfand, J. Sninsky & T. White (eds.), PCR Protocols: A Guide to Methods and Applications. pp. 315–322. Academic Press, San Diego.

Yoshida M., H. Hayakawa, T. Fukuda & J. Yokoyama. 2013. Incongruence between morphological and molecular traits in populations of *Viola violacea* (Violaceae). in Yamagata Prefecture, northern Honshu, Japan. Acta Phytotax. Geobot. 63: 121–134.

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